

STUDIES ON THE PHYSIOLOGICAL SIGNIFICANCE
OF CERTAIN PRECIPITATES FROM THE
EGG SECRETIONS OF ARBACIA
AND ASTERIAS

BY

ALVALYN E. WOODWARD

Simmons College

A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in the
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TWO CHARTS AND THREE FIGURES

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I. INTRODUCTION

A new phase in the physiology of fertilization practically began in 1912 when F. R. Lillie discovered the effect of egg extracts and secretions on sperm. He found that sea-water in which Arbacia eggs had been standing caused sperm of the same species to be activated, directed, reversibly agglutinated, and finally paralyzed. If a drop of this egg-sea-water is introduced, by means of a

capillary pipette, into a suspension of sperm, the latter become more active and gather around the drop as if chemically attracted. In addition, they form small clusters, 'agglutinations,' which last for several seconds. After this the sperm separate. The period of agglutination depends upon the activity and density of the sperm suspension and the strength of the egg-water. The effective material in the water Lillie ('12) called an iso-agglutinin.

The eggs of *Nereis* were found to produce a similar substance, which seemed to appear at the moment of fertilization. In this case, however, the active substance is destroyed by boiling, while *Arbacia* agglutinin is not entirely destroyed when boiled for seventy minutes. Lillie also found that an extract of *Arbacia* eggs agglutinated *Nereis* sperm. Because of differences in lability, he concluded that the *Arbacia* egg produced both iso- and hetero-agglutinins. He also discovered that if sperm be added in quantity to egg-water, the agglutinin is 'fixed,' and no longer affects sperm subsequently added.

For purposes of quantitative comparison, Lillie prepared a 'standard solution' of egg secretion by allowing one volume of 'dry' ripe eggs to stand in two volumes of sea-water for ten minutes. During this time the eggs were occasionally agitated. They were then precipitated with the centrifuge, and the supernatant fluid, carefully decanted, was subsequently used (Glaser, '14 c, p. 388). Since this secretion had such peculiar effects on spermatozoa, and since eggs from which it had been removed by repeated washings could not be fertilized Lillie has since called it 'fertilizin' ('13 b).

A substance to which the cell membrane is not permeable was also found in the egg. This material could be obtained only by laking with distilled water or crushing. Since this material was able to neutralize the agglutinative power of fertilizin, Lillie called it 'anti-fertilizin.' Antifertilizin does not prevent the chemotactic effect of egg secretion.

In addition, Lillie likewise discovered that *Arbacia* blood inhibits fertilization in the same species, but does not affect the agglutinative power of fertilizin. Moreover, the inhibitory effect of blood can be counteracted by an excess of fertilizin.

From these facts, Lillie deduced his 'side chain' theory of fertilization, in which he applied Ehrlich's conceptions to the subject at hand. He postulates that spermatozoa are chemically unable to unite directly with the egg, but that a third body, or amboceptor—in this case, fertilizin—is necessary to join them. Fertilizin, he believes, has two side chains, an ovophile group which can unite with a side chain of the egg, the egg receptor, and a spermophile group, which can unite with a sperm receptor. Fertilization may be blocked by 1) absence of the amboceptor; 2) occupation of either side chain by a foreign body; 3) occupation by similar means of either sperm or egg receptor. Further, "The sperm activates the fertilizing substance (fertilizin) already present in the egg. The egg is self-fertilizing (Lillie, 14, p. 587)."

II. CONCERNING THE FACTUAL BASIS OF THE FERTILIZIN THEORY

The fertilizin theory is founded upon three essential elements: an amboceptor, an egg receptor, and a sperm receptor. The presence of the amboceptor, Lillie believes, is indicated by a sharply defined reaction-agglutination of the sperm. This reaction is considered ('15) only as a visible, but not essential, symptom of a fundamental physicochemical change in the spermatozoa themselves. It is possible that in many species the egg secretion produces a significant change in the sperm without agglutinating them. The presence of sperm receptors, according to the same author, is indicated, among other things, by the Godlewski phenomenon, in which the inhibitory effect of foreign sperm is explained as due to the occupancy of the normal sperm receptors. The presence of an egg receptor he has been unable to demonstrate directly. It is, however, indicated by other inhibitors which effectively block the fertilization reaction.

A. Concerning egg secretions in general

The observations of Lillie with regard to the egg secretions of *Nereis* and *Arbacia* have been extended by several workers upon the same and other forms.

Just ('15 a) found that it was difficult or impossible to initiate development in *Nereis* eggs which had been washed. The eggs of *Platynereis*, also, are very sensitive to an excess of sea-water ('15 b). From these facts he concluded that a substance formed by the egg and necessary for development had been removed.

Fuchs found that the fertilizing power of the sperm of *Ciona*, *Arbacia pustulosa*, *Ascidia mentaul*, and *Strongylocentrotus lividus* could be increased by treatment with egg secretions of the same species. Moreover, the fertility of *Ciona* sperm could be enhanced by treatment with the egg secretion of *Phallusia*, *Arbacia*, or *Strongylocentrotus*. Likewise, *Strongylocentrotus* sperm are made more effective by the secretions of *Ciona*, *Echinus*, *Sphaerechinus*, and *Asterias* eggs. The egg extract, obtained by crushing the eggs, had the same effect as the secretion, except in the case of *Asterias*. In this instance, the secretion stimulates, while the extract poisons, *Strongylocentrotus* sperm. The method followed by Fuchs prevented the observation of sperm agglutination. He concluded that the secretions affected sperm only.

Asterias eggs, Glaser found ('14 b), form a secretion similar to that of *Arbacia*, except in color and a few minor points. Moreover, *Asterias* sperm are directed, activated, agglutinated, and paralyzed as well by *Arbacia* secretion as by that from their own species. *Arbacia* sperm react similarly to *Asterias* secretion. The writer has been able to confirm these observations and to demonstrate the reactions to colleagues.

While mature *Arbacia* eggs in a healthy condition are easy to obtain and always secrete a sperm agglutinin, the conditions differ in the case of *Asterias*. It was difficult, during the summers of 1915 and 1916, to obtain *Asterias* which contained many fertilizable eggs. In a good batch 10 per cent would produce larvae. Therefore, it was difficult to obtain the agglutinin in any usable amount. In June, 1914, however, *Asterias* at Woods Hole were in the best condition I have ever known. It was almost impossible to keep them in the laboratory three or four hours without their shedding eggs or sperm. Mr. Gray reported that the live-cars in which they were kept, though very open, were always

slimy with eggs. At that time, when 98 per cent to 100 per cent of the eggs would develop normally, most of the observations on the sperm agglutinin were made. All of them, however, were repeated and confirmed in 1915, 1916, or 1917.

As observed by Glaser, if a drop of sea-water, which had been standing over mature *Asterias* eggs, was injected under a cover-glass into a drop of sperm suspension, the sperm would gather into small, irregularly angular clusters of six to eighteen and remain agglutinated for a number of seconds which varied with the strength of the solution. When they separated, they were much more active than before. The general process of agglutination and activation is the same in *Asterias* as in *Arbacia*, where it has been observed by many. The chief difference is in the size of the clusters, which are smaller in *Asterias*.

As is known, *Asterias* eggs are obtained with the germinal vesicle still intact. Maturation changes begin almost immediately and are complete in forty-five to sixty minutes. Directly after obtaining them, some of the eggs of a large female were put into a centrifuge tube with two volumes of water and allowed to stand ten minutes to obtain fertilizin in the standard way. After centrifuging, the supernatant fluid was tested for its agglutinative power. When diluted 1 : 200, *Asterias* sperm remained agglutinated six seconds, a unit reaction. An hour after shedding, the rest of the eggs from the same female, which had been kept in a large amount of water, were washed twice with fresh sea-water, and then the secretion obtained as before. This, when diluted 1 : 200, caused a fresh sperm suspension to remain agglutinated over four minutes. In other words, it was over sixty times as strong as the other. Experiments of Lillie and Just indicate that in *Arbacia* and *Nereis*, as well as in *Asterias*, fertilizin is formed by the maturing or mature, but not by immature eggs. I believe that Lillie's failure (quoted by Loeb, '16, p. 83) to verify the observations with *Asterias* secretion was due to abnormal material.

Loeb ('15) has observed activation of sperm of the echinoderms at Pacific Grove by eggs of the same and related species. He also noted that the sperm of *Strongylocentrotus purpuratus* are

agglutinated by the egg secretion of the same species, while sperm of *S. franciscanus* are agglutinated not only by secretion from eggs of the same species, but also by that of *S. purpuratus*.

Miss Margaret V. Cobb (unpublished) discovered that the eggs and egg-water of *Cumingia* produce positive chemotactic response on the part of the sperm.

These results are summarized in table 1 and warrant the assertion that the eggs of at least four phyla of marine animals secrete into the sea-water substances significant either in their effects on sperm or in fertilization.

B. Concerning the secretions of Asterias and Arbacia in particular

While these facts indicate that the egg secretion may play an important rôle in fertilization, they do not make clear either the nature of the secretion itself or the manner in which it operates. It may be one homogeneous substance or a mixture of two or more specific substances.

Conceivably, there are three ways in which the problem can be attacked: 1) by physiological analysis of the secretion; 2) by general chemical analysis; 3) by the removal of specific elements from the secretion and the further analysis of their individual properties.

1. *Physiological analysis.* a. The secretion is necessary for fertilization in some species. As stated above, Lillie gave the name 'fertilizin' to the egg secretion because he considered its presence absolutely necessary for the fertilization of *Arbacia* eggs. Just found it equally indispensable for the fertilization of *Nereis* and *Platynereis*.

Loeb ('15) criticised their conclusion on the ground that the washed eggs had stood so long after shedding that they were dead, but the following experiment disposes of this objection. The eggs from ripe starfish were divided into two lots. One lot, A, was put into a finger-bowl of sea-water as control. The rest were placed into a large glass tube, closed at the bottom with chamois skin. The upper end was similarly closed except for an opening into which fitted closely a smaller tube through which

TABLE 1

SPERM OF THE FOLLOWING SPECIES															
WHEN TREATED WITH THE EGG EXTRACT OR SECRETION OF	Arbacia punctulata	Arbacia pustulosa	Ascidia mentula	Asterias forbesii	Asterias ochracea	Asterina	Ciona intestinalis	Cumingia	Echinus microtuberculatus	Nereis limbata	Platynereis megalops	Strongylocentrotus franciscanus	Strongylocentrotus lividus	Strongylocentrotus purpuratus	
Arbacia punctulata	Activated, aggregated, agglutinated, paralyzed (Lillie, Glaser, Woodward)			Activated aggregated, agglutinated, paralyzed (Glaser, Woodward)						Agglutinated (Lillie)					
Arbacia pustulosa		Become more fertile (Fuchs)					Become more fertile (Fuchs)								
Ascidia mentula			Become more fertile (Fuchs)												
Asterias forbesii	Activated, agglutinated. (Glaser, Woodward)			Activated aggregated, agglutinated (Glaser, Woodward)											

TABLE 1—*Concluded*

WHEN TREATED WITH THE EGG EXTRACT OR SECRETION OF	SPERM OF THE FOLLOWING SPECIES									
	Arbacia punctulata	Arbacia pustulosa	Ascidia mentula	Asterias forbesii	Asterias ochracea	Asterina	Ciona testudinis	Cumingia	Echinus microtuberculatus	Nereis limbata
Asterias glacialis										
Asterias ochracea										
Asterina										
Ciona intestinalis										
Cumingia										
Echinus microtuberculatus										

Nereis limbata	No effect (Lillie)								Agglutinated (Lillie) Secretion necessary for fertilization (Just)				
Phallusia mamillata					Become more fertile (Fuchs)								
Platynereis megalops									Egg-secretion necessary for fertilization (Just)				
Sphaerechinus granularis										Become more fertile (Fuchs)			
Strongylocentrotus franciscanus					No effect (Loeb)	Slightly activated (Loeb)				Activated, agglutinated (Loeb)		Activated, not agglutinated (Loeb)	
Strongylocentrotus lividus									More cleavage, stimulates hybridization (Fuchs)		Activated, attracted. More fertile (Fuchs)		
Strongylocentrotus purpuratus					No effect ² (Loeb)	Slightly activated (Loeb)					Activated, agglutinated (Loeb)		Activated, agglutinated (Loeb)

sea-water trickled slowly. The eggs were washed in running water in this manner for seventeen hours. These washed eggs were then divided into lots *B* and *C*. To *B* fresh egg secretion was added. All three were subsequently inseminated. In spite of the time which had elapsed since the eggs had been shed, a large number of the control, *A*, formed membranes. About the same number formed membranes in *B*, the washed eggs to which the secretion had been added, but in *C*, which was practically free from fertilizin, none was formed. None of the eggs developed beyond the eight-cell stage, doubtless due to the long time that had elapsed between maturation and insemination. The addition of egg secretion had restored the washed eggs to a fertilizable condition.

If, at the height of the breeding season, the eggs of *Asterias* or *Arbacia* are inseminated after the addition of fertilizin, the proportion developing is not materially affected. However, at the beginning or end of the season, a large part of the eggs are resistant to sperm fertilization. If fertilizin be added at that time, it will help to overcome this resistance as shown below:

TABLE 2¹

The effect of adding fertilizin to normal and to resistant eggs

DATE	ARBACIA EGGS + SPERM	EGGS + FERTILIZIN + SPERM
	<i>per cent cleavage</i>	<i>per cent cleavage</i>
July 2.....	72	68
	73	73
	84	75
August 9.....	24	86
	47	88

The result might be due to an increased permeability, since Glaser found that fertilizin has such an effect. It is very likely, however, that the resistance is due to lack of fertilizin, since egg

¹ Throughout the experiments, percentages have been reckoned from counts of at least two hundred normal-appearing eggs.

secretion obtained August 11, 1914, tested to only 50-units strength while that obtained by exactly the same method early in the season averaged 1600 to 3200 units and frequently ran higher.

b. The secretion, as stated on page 45, activates the sperm, attracts, reversibly agglutinates, and may finally paralyze them.

c. The secretion has equally striking effects on the egg. Glaser ('14 c) observed that *Arenicola* larvae treated with egg secretion lose pigment, indicating an increase in permeability. Taking his cue from this, and from the fact that parthenogenetic agents as a class increase permeability, he found that when the secretion derived from either *Asterias* or *Arbacia* was added to ripe eggs of the same species, and allowed to act on them for two hours, the eggs began to segment. To this process he gave the name 'autoparthenogenesis.' Following the method of Loeb ('13, p. 70), Glaser found that after-treatment with hypertonic sea-water (50 cc. sea-water and 8 cc. 2.5 m. NaCl) increased the percentage of developing eggs.

Glaser's work on autoparthenogenesis was easily confirmed. The 'dry' ripe eggs of *Asterias* were placed in watch-glasses with at least two volumes of standard fertilizin for one and one-half to three hours. They were then rinsed two or three times with sea-water, and treated with hypertonic sea-water (50 cc. sea-water and 8 cc. 2.5 m. NaCl) for a suitable length of time. After rinsing with sea-water, they were left to develop. It is better to transfer them to finger-bowls with a quantity of sea-water (table 3). The optimum time for the exposure of the eggs to fertilizin and for the hypertonic after-treatment seemed to vary with the temperature and with the batch of eggs. Occasionally the entire lot of eggs would give normal cleavage and normal gastrulae. More often, however, cleavage of both nucleus and cytoplasm was irregular, as described by Wilson ('01) for parthenogenetic eggs. If, when the cytoplasm first divided, the nucleus went entirely into one half, the other half would disintegrate and only the nucleated part would develop into a ciliated blastula. Consequently, it was a frequent occurrence to find a small *Asterias* blastula swimming around among cytoplasmic fragments within

the fertilization membrane. Practically the same thing occurred in the case of Arbacia, with the exception that no fertilization membrane was formed, and only the jelly surrounding the egg held the fragments together for a time. This soon broke up, however, and allowed the larvae to escape. The irregularity of cleavage was less marked in the eggs which had not been subjected to hypertonic after-treatment, but, on the other hand, the eggs with the hypertonic after-treatment gave a larger per-

TABLE 3
Autoparthenogenesis in Asterias eggs

TREATED WITH FERTILIZIN	HYPERTONIC SEA-WATER	PER CENT CLEAVAGES	REMARKS
<i>hours</i>	<i>minutes</i>		
1½	0	50	1 blastula, cleavage normal
1½	40	42	Cleavage normal
2	0	50	
2	20	57	Few ciliated
2	30	46	Many ciliated
2	45	41	Few ciliated
2½	0	52	None ciliated
2½	20	66	Nearly all ciliated
2½	30	63	Few ciliated
2½	45	69	None ciliated
3	0	66	None ciliated
Sperm control.....		74	
Untreated control.....		0	

centage of swimming larvae. That is, these eggs developed farther than those treated with fertilizin alone.

While autoparthenogenesis must be considered in formulating any general theory of fertilization, it is also of immediate practical value in our analysis of the egg secretion, which is thus shown to affect both egg and sperm. This indication that the secretion may have at least a dual nature is strengthened by other observations: 1) Boiling does not destroy its power to agglutinate sperm (Lillie), but does destroy its value as a parthenogenetic agent (Glaser). 2) Arbacia blood inhibits sperm fertilization, but does not affect the agglutinating power of the secretion (Lillie);

in other words, Arbacia blood has no effect on the 'spermophile group' (Lillie). 3) Loeb ('14) dissolved the jelly from the eggs of *S. purpuratus* by means of $\frac{N}{10}$ HCl. These eggs were no longer able to agglutinate sperm, but could be fertilized. From this experiment and from the fact that some species can be fertilized by sperm of other species, even though those sperm are not agglutinated by secretions from the eggs, Loeb concluded that "The substance which is responsible for cluster-formation is not necessary for the process of fertilization." Lillie ('15) repeated this experiment, using *Arbacia* instead of *Strongylocentrotus*, and found, as soon as the acid was entirely washed away, that the eggs continued to secrete an agglutinin. It is possible that in Loeb's experiment the agglutinin was so dilute as to give only a momentary reaction which was overlooked or that the presence of acid interfered with the reaction.

2. *General chemical properties of the egg secretion.* a. Properties shown by qualitative chemical tests. Glaser ('14 b) undertook a qualitative analysis of both *Arbacia* and *Asterias* secretions. He found them neutral to litmus and unable to reduce Fehling's solution. Various tests for protein failed to give a distinct reaction, although there were indications that protein might be present in very small amounts. For instance, while the "xanthoproteic test gave no precipitate, the solution turned distinctly yellow." He was also unable to obtain a precipitate either by boiling or by adding alcohol.

The writer confirmed all of these points. It was also found that the secretion does not dialyze through a collodion sac. Benedict's test for sugar was applied, as being more sensitive than Fehling's, but gave negative results. Since the xanthoproteic test produces a yellow color, we may infer the presence of tyrosine, phenylalanine, or tryptophane.

It was not deemed advisable to carry out a complete chemical analysis of the secretion. Qualitative tests, however, indicated the presence of both carbon and nitrogen.

b. Properties shown by x-radiation. In 1914 the writer was able, coöperating with A. Richards, to study the effect of x-radiation on *Arbacia* egg secretion. An account of these experiments,

though already published (Richards and Woodward), may be summarized here. Equal amounts of the secretion were placed in four open dishes. One was kept shielded from x-rays for a control, one was radiated two minutes, another five minutes, and the third, fifteen minutes. The agglutinating power of each was then tested with fresh sperm suspension, by finding the dilution necessary to bring about a unit reaction. It was found that the secretion which had been radiated two minutes had the greatest agglutinating power. That which had been radiated five minutes was about the same as the control, while radiation for fifteen minutes decreased the efficiency of the agglutinin. These

TABLE 4
The effect of x-radiation on the parthenogenetic power of fertilizin

	PER CENT CLEAV- AGES	PER CENT NORMAL BLASTULAE	PER CENT CLEAV- AGES	PER CENT NORMAL BLASTULAE	PER CENT CLEAV- AGES	PER CENT NORMAL BLASTULAE	PER CENT CLEAV- AGES	PER CENT NORMAL BLASTULAE	PER CENT CLEAV- AGES	PER CENT NORMAL BLASTULAE
Sperm control.....			23.8		46.6					
Fertilizin control (unradiated)....	30.2	0	9.5	0	5.2	1	20.2	.5	15.5	0
Fertilizin 2 minutes radiation....	24.3	Few	14.1	1	10.8	3	13.5	0	28.5	2
Fertilizin 5 minutes radiation....	21.6	Few	17.9	0	8.4	0	10.5	0	87.2	0
Fertilizin 15 minutes radiation...	17.5	0	15.6	0.5	9.5	0	13.6	0	52.1	0

results correspond closely with those previously obtained by Richards ('14) when enzymes were radiated, and suggest very strongly that sperm agglutinin may also be an enzyme.

The effect of radiation of the agglutinin wears off in two or three hours, the length of time during which eggs should be exposed to the secretion in order to produce autoparthenogenesis. If the effect on the egg activator is equally transitory, one would not expect that radiation would greatly affect its parthenogenetic power. Experiments 3 and 5, table 4, suggest, however, that the effect of radiation on the parthenogenetic power of fertilizin is similar to that on its agglutinating power.

c. The relation between concentration and agglutinating power. The work of 1914 (Richards and Woodward) also brought out

the fact that "the efficiency of the agglutinin contained in fertilizin, like pepsin (Euler, p. 132), varies with the square root of the concentration." The efficiency may be measured by the length of time the sperm remain agglutinated and the concentration by Lillie's units of strength (Lillie, '14). Curves may be plotted by letting x equal the concentration and y the efficiency. The average of twelve such curves gives a figure which corresponds very closely to the curve of the equation $y^2 = 11x$, or, $y = c\sqrt{x}$ (fig. 1). The curves are nearly identical in the lower concentrations, where experimental determination is more accu-

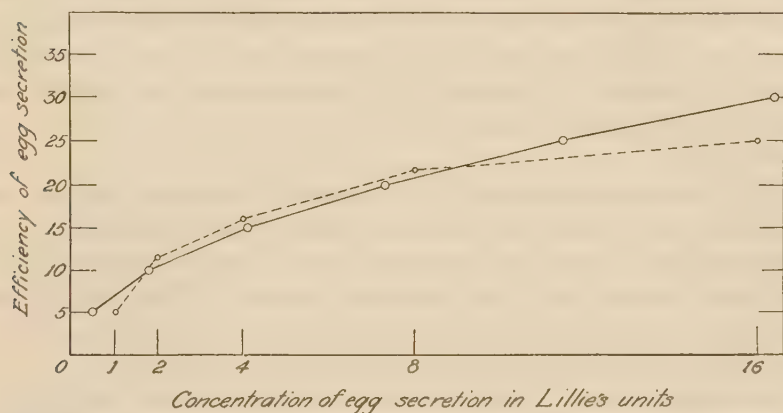


Fig. 1 The solid line is the curve of the equation $y = c\sqrt{x}$. The broken line represents the results of twelve experiments.

rate and where the greater number of values is averaged. They coincide in all parts within the limits of experimental error. It is safe to conclude, therefore, that the sperm agglutinin resembles enzymes in the relation between efficiency and concentration.

d. Tests for enzymes. If, as the radiation tests suggest, enzymes are present, it seemed likely that we might be dealing with oxidase or catalase or both. The former gives a blue reaction with guaiac, especially if hydrogen peroxide is added. If *Arbacia* secretion made from fresh eggs is tested with guaiac plus H_2O_2 , no blue color appears. If the secretion is made from eggs which have stood for some time, so that some of them are cytolysed,

an oxidase reaction is given. It seems, therefore, that the oxidase present within the egg cannot permeate the normal membrane, and is not present in 'pure' fertilizin.

That catalase is not present in the secretion unless the eggs have been laked or cytolized is indicated by the fact that such an extract does not free oxygen from hydrogen peroxide (method of Loevenhart, '05). The eggs themselves, however, contain a considerable amount of this enzyme, which, like the oxidase, cannot permeate the normal membrane. This was also observed by Amberg and Winternitz ('11).

No evidence of the presence of a proteolytic enzyme could be demonstrated by the digestion of egg albumin by either fertilizin or a precipitate obtained from the secretion. This, of course, does not preclude the possibility of the presence of an enzyme that splits some *Arbacia* protein, but is powerless against that of a hen's egg.

While it was not expected that the secretion would contain an amylase, a mixture of fertilizin and boiled starch was allowed to stand several hours. At the end of that time, Benedict's test for sugar was applied with negative results. The experiment was also performed with a precipitate obtained from the secretion instead of fertilizin, but with negative results as before.

The only enzyme test with positive results was that for a lipase. In order to execute this test, a fatty substance was extracted from a large quantity of *Arbacia* eggs which had been freed from water as completely as possible by centrifuging and pipetting. The fat in these eggs was extracted with two or more volumes of ether. Later, this fatty extract, when shaken with water, formed an emulsion in which the fat globules were quite small. By measuring these in a hanging drop with a micrometer eye-piece, their diameters were found to be constant for three hours. Fresh egg secretion in which the lipase might be presumed to be present had no measurable effect on the size of the drops. It would be a mistake to conclude, however, that lipase is absent. By a modification of Robertson's method (p. 475) it is possible to throw down a precipitate from egg secretion soluble in water and capable of dissolving the oil. The

reaction with this precipitate takes place rather quickly. At the end of the two and a half hours it was found that the decrease in diameter of the drops amounted to between 10 per cent and 23.5 per cent, with an average of 16.7 per cent. This would indicate that the secretion could change the fat into a compound soluble in water.

3. *The precipitation of specific elements from the egg secretion and their properties.* Of especial significance is the study of substances precipitated from egg secretions. It will be recalled that neither Glaser nor the writer obtained a precipitate from the egg secretion by boiling or adding absolute alcohol. Since, however, the secretion was colloidal (not dialyzable), other methods of precipitation were tried in 1915 and 1916.

a. *Precipitation of a sperm agglutinin.* If the egg secretion of *Arbacia* is saturated with crystals of pure $(\text{NH}_4)_2\text{SO}_4$, a whitish flocculent precipitate is formed. This may be washed in saturated ammonium sulphate solution and dissolved in either fresh or sea-water. The solution causes reversible agglutination of sperm, while ammonium sulphate alone paralyzes but does not agglutinate them. If the precipitate is dissolved in distilled water and dialyzed in a collodian sac against distilled water or running tap water until, as shown by testing with BaCl_2 , all the SO_4 is removed, it still causes reversible agglutination of sperm. This power is lost after a few days, probably because of bacterial action. The precipitate may, however, be kept ten days or more without losing its agglutinative power if covered by saturated $(\text{NH}_4)_2\text{SO}_4$, which prevents the growth of bacteria. By this method a solution may be obtained with greater agglutinating power than the original egg extract. The method does not, however, bring about complete precipitation of the sperm agglutinin, as was shown by dialyzing and testing the filtrate obtained after the addition of $(\text{NH}_4)_2\text{SO}_4$. This still caused agglutination. This precipitate has no parthenogenetic power.

b. *Precipitation of a lipolysin.* Since there were strong indications of the presence of lipase and perhaps other enzymes, I adapted a method used by Robertson ('12) to obtain an enzyme from blood serum. Eight volumes of fertilizin and four volumes

of 7 per cent BaCl_2 were kept at 37°C . for an hour or more and stirred frequently. The mixture was then centrifuged and the filtrate discarded. The precipitate was washed several times with BaCl_2 and then treated with N/10 HCl . To this solution was added Na_2SO_4 in excess to precipitate the barium, and the mixture was allowed to stand overnight. The liquid was then centrifuged and to the clear fluid were added four to five volumes of acetone, which caused a heavy flocculent precipitate. This was filtered, and the precipitate was washed several times with absolute alcohol² and ether, and then dried for thirty-six hours or more over H_2SO_4 . The resulting powder dissolves readily in both sea-water and distilled water. It should be added that this precipitate was invariably obtained with good fertilizin and freshly distilled acetone during the summer of 1915 and until about the middle of July, 1916. It was never obtained by adding acetone to sea-water. By the middle of 1916, all of the recently purchased acetone had been used up, and recourse was necessary to some purchased from Kahlbaum in 1912. This was yellowish in color, slightly acid to litmus, and differed slightly in odor from the fresh. With this acetone, no precipitate was obtained excepting after adding NaOH , and even then only in traces.

Since (p. 474) the precipitate dissolves a fat extracted from the egg, we may call its active principle 'lipolysin.' In solution it does not agglutinate *Arbacia* sperm. It is, however, a very efficient parthenogenetic agent (table 5).

The solution used in these experiments contained about $\frac{1}{4}$ cc. of loose powder (about 0.025 grams) to 10 cc. sea-water. A much larger percentage of normal larvae was obtained by this precipitate than I have ever been able to obtain by any other artificial method.

It should be noted in this connection that, while the lipolysin can be precipitated from mature *Asterias* eggs, it has been impossible to obtain any acetone precipitate from immature ones. About 100 cc. each of *A*, immature, resistant starfish eggs and of

² The failure to obtain a precipitate with alcohol (p. 471) was due to its dilution. While this precipitate is insoluble in absolute alcohol, it dissolves readily in 75 per cent.

B, eggs of which 50 per cent or more matured, were treated side by side, to obtain the egg extract and to precipitate it. The precipitate from *A* was so slight that it produced only a faint cloudiness. That from *B*, when dried, nearly filled a Syracuse watch-glass.

A sample of lipolysin prepared by this method from *Arbacia* eggs in August, 1915, was examined and tested in June, 1916. Its color had changed from white to a grayish violet, it had become compact, and it was much less soluble than when first made. Its efficiency as a parthenogenetic agent will be seen in table 6. Other samples of the precipitate, now a year old, do not show the physical changes noted in the 1915 sample.

TABLE 5
Lipolysin as a parthenogenetic agent

Eggs from lot.....	PERCENTAGE OF CLEAVAGES					
	A ¹	B	C	D	E	F
Control, unfertilized.....	0	0	0	0	0	0
Eggs + sperm.....	99	99	99	95	18	81
Eggs + hypertonic 20 minutes.....				0	0	0
Eggs + hypertonic 30 minutes.....				4	7	0
Eggs + lipolysin 2 hours + hypertonic 30 minutes...				20	17	7
Eggs + lipolysin 170 minutes + hypertonic 20 minutes.....	31	44	53			

¹ In this and in the following tables a vertical column represents the results from the eggs of a single female or from a thoroughly mixed batch of eggs.

In 4*B* practically all the eggs which divided developed into swimming larvae. In 6*A*, 12 per cent swam; in 6*B*, 10 per cent, and in 8*A* also a number swam. It will be noted that fresh *Asterias* lipolysin is more efficient with *Arbacia* eggs than is the old *Arbacia* precipitate.

This parthenogenetic development was not caused by impurities, for the lipolysin does not contain a trace of barium, as proved by careful spectroscopic tests; neither does it contain acetone, alcohol, or ether, all of which were carefully removed.

Both the lipolysin and sperm agglutinin may be obtained from the same sample of egg secretion. In order to do this, BaCl₂ is added first, and the precipitate saved for extraction of the lipo-

lysin. To the filtrate Na_2SO_4 is added in small quantities until all the excess of barium is precipitated. The liquid remaining can be saturated with $(\text{NH}_4)_2\text{SO}_4$ to precipitate the sperm agglutinin.

The work outlined on page 470 suggests that the egg secretion consists of at least two substances. They have now been separated, and hence we may consider the sperm agglutinin or

TABLE 6
Lipolysin not specific as parthenogenetic agent

	PERCENTAGE OF CLEAVAGES ¹	
	A	B
1. Arbacia eggs, control.....	0	0
2. Arbacia eggs + sperm.....	96	97
3. Arbacia eggs + Asterias lipolysin 1 hour.....	6	8
4. Arbacia eggs + Asterias lipolysin 1 hour + hyper 20 minutes..	5	50
5. Arbacia eggs + Asterias lipolysin 1½ hours.....	20	15
6. Arbacia eggs + Asterias lipolysin 1½ hours + hyper 20 minutes.....	60	25
7. Arbacia eggs + Asterias lipolysin 2 hours.....	15	20
8. Arbacia eggs + Asterias lipolysin 2 hours + hyper 20 minutes..	60	0
9. Arbacia eggs + Arbacia lipolysin 1 hour.....	10	10
10. Arbacia eggs + Arbacia lipolysin 1 hour + hyper 20 minutes..	12	5
11. Arbacia eggs + Arbacia lipolysin 1½ hours.....	1	0
12. Arbacia eggs + Arbacia lipolysin 1½ hours + hyper 20 minutes.....	10	5
13. Arbacia eggs + Arbacia lipolysin 2 hours.....	25	5
14. Arbacia eggs + Arbacia lipolysin 2 hours + hyper 20 minutes	40	15

Note.—The solution of lipolysin was made by dissolving 2 cc. of the powder, shaken down in the bottom of a graduated centrifuge tube, in 15 cc. sea-water. Nos. 3 to 8, inclusive, were made with freshly precipitated Asterias powder (one week old); Nos. 9 to 14, with Arbacia precipitate ten months old.

¹ Estimated.

spermophile group, as distinct from the lipolysin, or ovophile group. The reason for Lillie's belief that fertilizin is an amboceptor is now clear. The effect of x-radiation on fertilizin and the relation between efficiency and concentration of the secretion suggest that the sperm agglutinin may be an enzyme. There is slight evidence from these tests concerning the nature of the parthenogenetic agent. When we actually apply to the precipitates tests for the individual classes of enzymes, however, it

is the parthenogenetic portion of fertilizin which suggests a positive result—one indicative of a lipase. If the agglutinin is an enzyme, its nature is not yet known.

C. Concerning inhibitors

The dual nature of the secretion in no wise impairs the utility of inhibitors in the analysis of the fertilization reaction. An inhibitor may function in a purely mechanical or physical manner as by making the egg membrane tough so that the sperm cannot enter, or it may remove from the reaction system one or more of the essential reagents. Such an inhibitor, as Lillie ('14) has pointed out, may act by: 1) Removing fertilizin. 2) Occupying the 'sperm receptors.' 3) Occupying the 'spermophile side-chain' (agglutinin). 4) Occupying the 'ovophile side chain' (lipolysin). 5) Occupying the 'egg receptors.' In other words, an inhibitor may combine with the agglutinin, the lipolysin, or the substances within the sperm or egg with which these react. We shall consider for the present only inhibitors of organic origin.

1. *Removal of fertilizin.* Repeated washing is the only method yet devised for removing fertilizin. The discussion of the effects of this treatment, page 464, shows it to be an effective means of inhibition.

2. *Combination with sperm receptors.* Godlewski ('10) found that sea-urchin eggs to which had been added *Chaetopterus* sperm were made resistant to sperm of their own species subsequently added. Lillie interprets this as being due, perhaps, to a change in the spermatozoa which prevents their union with fertilizin. In other words, he believes that from *Chaetopterus* sperm may be dissolved into the water some substance which combines with the sea-urchin 'sperm receptor' and occupies the group which would normally unite with the spermophile side chain of fertilizin. The receptor so 'occupied' would be unable to combine with the fertilizin-egg complex, and therefore fertilization would not occur. This interpretation, Lillie admits, is purely hypothetical. No experiments to test it have been performed. It would be just as reasonable to assume that *Chaetopterus* sperm combined with or adsorbed the agglutinin or the lipolysin or that they in some way injured the egg itself.

3. *Combination with the 'spermophile group' (agglutinin) of fertilizin.* It will be recalled that (p. 460) 'anti-fertilizin' was obtained by Lillie by breaking up Arbacia eggs after most of the fertilizin had been removed through washing. He observed that a mixture of anti-fertilizin and fertilizin was unable to agglutinate sperm. The inhibition in this case he assumed to be due to occupancy of the spermophile group of the fertilizin.

4. *Combination with the 'ovophile group' (lipolysin) of fertilizin.* That anti-fertilizin inhibits sperm fertilization as well as agglutination, I found in experiments where the controls produced 77 per cent and 82 per cent cleavages, while the eggs treated with anti-fertilizin produced, respectively, 68 per cent and 21 per cent cleavages. It is even more efficient in blocking autoparthenogenesis in Arbacia (table 7).

TABLE 7

Inhibition of autoparthenogenesis by anti-fertilizin

	PERCENTAGE OF CLEAVAGES	
	A	B
Eggs + fertilizin 2½ hours + hypertonic NaCl 20 minutes.....	43	57
Eggs + antifertilizin + fertilizin 2½ hours + hypertonic 20 minutes.	10	0
Eggs + boiled anti-fertilizin + fertilizin 2½ hours + hypertonic 20 minutes.....	2	0

Since it prevents autoparthenogenesis, anti-fertilizin must combine with the parthenogenetic agent, lipolysin, or with the egg, as well as with the agglutinin. This versatility is not surprising when we remember that under the term 'anti-fertilizin' are grouped all the egg contents to which the membrane is not permeable. It must include the fatty substance with which lipolysin combines.

From the fact that this particular union with the parthenogenetic agent takes place, we should anticipate that the fatty substance itself would inhibit the development of the egg (table 8). To obtain the fatty extract, one volume of sea-urchin eggs and two or more volumes of ether were shaken and then allowed to settle into layers. The solution used consisted of 0.3 cc. of this ethereal extract diluted with 100 cc. sea-water. In experiment 3,

TABLE 8

Inhibition of cleavage by fat extracted from eggs

TREATMENT OF EGGS	PERCENTAGE OF CLEAVAGES					
	A	B	C	D	E	F
5 cc. eggs + sperm.....	86	85	100	100	100	100
5 cc. eggs + 1 cc. 0.3 per cent ether + sperm.....	90	94	99	100	100	98
5 cc. eggs + 1 cc. 0.3 per cent ether extract + sperm..	13	82	0	29	0	0
5 cc. sperm suspension + 1 cc. 0.3 per cent ether extract + eggs.....	19	2	13	12	3	14

table 8, 5 cc. of Arbacia eggs and sea-water were allowed to stand with 1 cc. of this solution ten minutes before the addition of the sperm. In 4, the sperm stood in the solution ten minutes before eggs were added. In both cases, fertilization was inhibited. Tests with iodine show that this extract contains an unsaturated fatty acid, probably similar to that which forms about 30 per cent of the phosphatide in Asterias eggs (Mathews, '13).

Lillie found ('14) that Arbacia blood likewise blocks sperm fertilization in the same species. My confirmatory experiments are given in table 9. The 'serum' used was obtained by filtering the perivisceral fluid, after it had stood long enough to clot. It will be noted that boiling the serum sometimes removes the inhibitor.

Since Arbacia serum does not affect the agglutinating power of fertilizin and since its ability to inhibit development of the egg can be overcome by excess of fertilizin, Lillie concluded that block in this case was produced by occupancy of the ovophile group. The following experiment shows clearly that the sperm is not affected in this block. Arbacia eggs were left standing in blood of the same species for fifteen minutes. Then they were washed

TABLE 9

Inhibition of cleavage by Arbacia serum

	PERCENTAGE OF CLEAVAGES						
	A	B	C	D	E	F	G
Arbacia eggs + sperm.....	40	79	93	72	47	45	24
Arbacia eggs + Arbacia serum + sperm.....	0	12	0	1	5	0	0
Arbacia eggs + boiled serum + sperm.....				69	1	3	43

repeatedly with sea-water and inseminated. In one case the control showed 79 per cent cleavages and the experimental eggs 12 per cent. In another case the cleavages were 40 per cent and 0, respectively. In both dishes the sperm appeared active and normal.

There seems to be no definite proof concerning the mode of action of this inhibitor. Since mammalian blood contains specific substances which prevent autolysis, we might expect echinoderm serum to contain similar substances. These should, theoretically, prevent the action of the egg enzymes and might or might not resemble the fatty inhibitor already in the egg. If they are fatty, they would probably combine with lipolysin as Lillie suggested, otherwise they might not.

TABLE 10
Inhibition of cleavage in Asterias by Asterias and Arbacia sera

	PERCENTAGE OF CLEAVAGE	
	A	B
Asterias eggs + sperm.....	30	28
Asterias eggs + Asterias serum + sperm.....	0	0
Asterias eggs + Arbacia serum + sperm.....	0	0

The fact that Arbacia serum inhibits sperm fertilization in eggs of the same species suggested trying it with Asterias eggs, and also suggested that Asterias serum might likewise contain an inhibitor. The experiments shown in table 10 prove the suggestion correct.

5. *Combination with egg receptors.* 'Purple x', discovered by Glaser ('14 c), is a purple compound which appears when Arbacia sperm or egg secretion are boiled. He found that the material prevents both normal fertilization and autoparthenogenesis. Since it does not prevent the agglutination reaction of fertilizin, Glaser inferred that it did not act by combining with sperm receptors or with agglutinin. It might, however, combine with either the lipolysin or the egg. No decisive experiments have been performed as yet. Since the jelly surrounding the egg becomes swollen and sticky, sperm fertilization may be prevented

TABLE 11
Inhibition of cleavage by purple x

	PERCENTAGE OF CLEAVAGES							
	A	B	C	D	E	F	G	H
Arbacia eggs + sperm.....	Polyspermy	47	24	Polyspermy	72	73	72	84
Arbacia eggs + boiled sperm (purple) + sperm.....	4			39	27	0	0	0
Arbacia eggs + boiled sperm (colorless) + sperm.....	93	63	61	82	77	55	68	

mechanically by entrapping the sperm. The egg itself also appears swollen, and hence may be so changed that it cannot develop. It will be noted in the following experiments (table 11) that boiled Arbacia sperm blocks fertilization only when 'purple x' is present. This inhibitor (Woodward, '15) appears to be derived from testicular or ovarian tissue.

Boiling the sperm or egg secretion of *Asterias* produces a salmon-colored substance, 'salmon x,' which also acts as an inhibitor with both *Asterias* and *Arbacia* eggs. The color of the boiled *Asterias* sperm suspension was not always noted. The inconsistent results shown in the second line of table 12 arouse the suspicion that in some cases the inhibitor, and perhaps the salmon-colored compound, was not present.

TABLE 12
Inhibition of cleavage by salmon x

TREATMENT OF EGGS	PERCENTAGE OF CLEAVAGES					
	A	B	C	D	E	F
<i>Asterias</i> eggs + sperm.....	75	14	25	37	30	28
<i>Asterias</i> eggs + boiled <i>Asterias</i> sperm + sperm....	78	66	17	41	3	21
<i>Asterias</i> eggs + boiled <i>Asterias</i> fertilizin + sperm...	13	1	6	21		
<i>Arbacia</i> eggs + sperm.....			73	72	84	
<i>Arbacia</i> eggs + boiled <i>Asterias</i> sperm + <i>Arbacia</i> sperm.....			0	0	0	
<i>Arbacia</i> eggs + boiled <i>Asterias</i> fertilizin + <i>Arbacia</i> sperm.....			0	0	0	

6. *Unclassified inhibitors.* Echinochrome, the red pigment coloring *Arbacia* 'blood cells,' completely inhibits the cleavage of *Arbacia* eggs. It is prepared by allowing *Arbacia* 'blood' to clot, washing the clot with sea-water to rid it of serum, and then laking the cells by adding a measured volume of distilled water. To make this solution isotonic with the eggs, it is necessary to add an equal volume of 'double sea-water,' i.e., sea-water boiled down to one-half its original volume.

It was suspected at one time that 'purple x' and echinochrome might be identical. MacMunn ('85) found that the addition of NaOH to echinochrome turned it yellowish, while HCl produced a red-yellow color. Since neither reagent produces a color change in 'purple x' (Woodward, '15), the suspicion has no foundation.

We may summarize the methods of inhibition as follows:

Foreign sperm (Godlewski, Herlant)...	Which occupy sperm receptors (Lillie).
Anti-fertilizin (Lillie).....	Which occupies the spermophile group (Lillie) and combines with lipolysin (Woodward).
Washing (Lillie).....	Which removes 'fertilizin' (Lillie).
Blood of same species (Lillie).....	Which occupies ovophile group (Lillie).
'Purple x' (Glaser).....	Which occupies egg receptor (Glaser) or causes physicochemical change in egg (Woodward).
'Salmon x' (Glaser).....	Ditto.
Blood of related species (Woodward)...	Which may combine with lipolysin (Woodward).
Fatty extract from eggs (Woodward)...	Which combines with lipolysin (Woodward).
Echinochrome (Woodward).....	Which appears to change egg itself (Woodward).

III. CONCERNING ACTIVATORS—PARTICULARLY LIPOLYSIN—AND THEORIES OF ACTIVATION

To the eye, 'activation' of an egg is manifested by cleavage. Side by side with this goes an acceleration of metabolism indicated by a three- to five-fold increase in the rate of oxidation in developing over resting eggs (Warburg, '08 and Loeb and Was-teney, '13). This increased metabolism is also found in cytolyzing eggs.

A. Artificial activation

In our attempts to understand activation, the method of imitating the effect of sperm by physical and chemical means has been of the greatest service. The first change to be noticed when an echinoderm egg is fertilized is the appearance of a 'fertilization membrane.' Such membrane formation may be brought about by a large number of agents (Loeb, '13, '16), distilled water, the lower fatty acids, lipid solvents, bases, certain salts, increased temperature, shaking in some cases, saponin, solanin, and bile salts. All of these cause cytolysis. If the cytolysis is confined to the cortical layer of the egg, a membrane will be formed and the egg will develop. Most of these agents, however, if left to themselves, cytolysed not only the cortical layer, but the whole egg. Loeb found it necessary, therefore, either to follow the cytolytic agent with treatment by hypertonic solutions in the presence of oxygen or to inhibit oxidation. The latter may be accomplished by depriving the eggs of oxygen or by treating them with a cyanide.

So far as is known, there are only three classes of physiological activators, the egg secretion of the same or related species, the blood-serum of unrelated species, and, finally, the spermatozoön. That the egg secretion may cause superficial cytolysis is indicated by the work of Glaser, who found that it increases the permeability of *Arenicola* larvae. Many investigators, notably Friedenthal, have found that mammalian blood-serum contains something which cytolyses cells of unrelated animals. Hence it is not surprising to find that foreign blood and extracts of foreign cells cause cytolysis and activation of Echinoderm eggs (Loeb, '13).

On the basis of these facts, Loeb ('12) concludes that "the spermatozoön, as well as the blood and tissues, contains a substance (lysin) which causes only cytolysis of the cortical layer." Such a theory brings the phenomena of parthenogenesis and sperm fertilization into one class.

F. R. Lillie, on the other hand, believes that the spermatozoon "functions essentially as the activator of a third body, 'ferti-

lizin,''' which, in turn, causes cleavage. Such an activation of fertilizin might be conceived as an increase in the affinity of the ovophile group for the egg receptor (Glaser). This hypothesis seems difficult to confirm. How are we to get rid of the sperm in a sperm-fertilizin mixture without injuring the fertilizin? Sperm pass readily through any available filter through which fertilizin can pass, but can be killed by heating to 40°C. for three minutes. Since fertilizin does not lose its ability to agglutinate sperm if boiled for a few minutes, a method suggests itself.³

Equal amounts of Arbacia fertilizin were put into two test-tubes and to one was added a large amount of Arbacia sperm. They were allowed to stand fifteen minutes, then both tubes were

TABLE 13

A comparison of the parthenogenetic effects of fertilizin with those of fertilizin 'activated' by sperm

	PER CENT CLEAVAGES
1. Eggs (Control 1).....	0
2. Eggs + fresh sperm (control 2).....	70.5
3. Eggs + heated fertilizin (diluted to $\frac{1}{2}$) 2 hours + hypertonic 20 min.	61
4. Eggs + heated fertilizin (diluted to $\frac{1}{2}$) 2 hours.....	22
5. Eggs + heated mixture of fertilizin and sperm (diluted to $\frac{1}{2}$) 2 hours	21
6. Eggs + heated fertilizin (diluted to $\frac{1}{4}$) 2 hours.....	0
7. Eggs + heated mixture of fertilizin and sperm (diluted to $\frac{1}{4}$) 2 hours	0

heated in a water-bath to 40°C. for three minutes. Eggs were then treated as in table 13. In experiment 4 and those following, hypertonic after-treatment was not given, since that in itself causes development in some cases, and I wished to learn the effect of the 'activated fertilizin.' Dilutions were used because it was thought that they might show more clearly the greater activity of fertilizin mixed with sperm. Repetition of this experiment gave essentially the same results.

Since the heated fertilizin alone caused development in 22 per cent of the eggs and that to which sperm had been added caused

³ Glaser first attempted to solve this problem, but failed because he boiled the mixture of sperm and fertilizin, and thus obtained 'purple x,' which inhibited development.

cleavage in 21 per cent, the indication is that the sperm does not activate the fertilizin. Of course, this is not conclusive, since the temperature which kills the sperm may also destroy the 'activated' fertilizin, and unless this is broken down into the unactivated form, we should expect fertilizin treated with sperm to be markedly weakened. Since this is not the case, we shall have to conclude that an activating effect on the ovophile group, or lipolysin, is undemonstrable. But why should we expect activation? The 'ovophile' and 'spermophile' groups are two independent substances. Such being the case, it is not surprising that a reaction involving one does not 'activate' the other.

Since Lillie's idea of activation cannot be accepted, we are brought back to superficial cytolysis and its connection with the activation of the egg. It is quite natural that superficial cytolysis should have been considered a cause of activation. On this assumption, the search for a *modus operandi* has been carried on by several investigators. Loeb himself suggests that cytolysis removes an obstacle to development. R. S. Lillie ('11) believed the obstacle might be CO_2 . Loeb proved that this was impossible by using sea-water charged with CO_2 as a parthenogenetic agent. Glaser suggested that the obstacle might be antifertilizin. Both R. S. Lillie and Glaser postulated that the removal of these obstacles was due to the increased permeability of the egg resulting from cytolysis.

But suppose that cytolysis is not the cause of activation, but an effect (of minor significance) of other processes which produce development? From this standpoint we get a different view of the situation.

The egg has been found to contain a considerable amount of lipoid, about 30 per cent of which is an unsaturated fatty acid. Overton and others have shown that this lipoid must be more concentrated at the surface than in the interior. According to Jobling, an unsaturated fatty acid inhibits enzyme action. We know that the egg contains a quantity of enzymes—catalase and oxidase, for instance—which control metabolism. Let us assume that the activity of the enzyme is decreased by the presence of the unsaturated fatty acid. Removal of this fatty acid,

then, should result in increased metabolism. With this assumption in mind, let us examine the methods of artificial parthenogenesis.

Lipoid solvents, such as butyric acid, chloroform, chloral hydrate, and ether, can remove the fatty inhibitor by dissolving it, and thus allow the enzymes to act more rapidly. Bases saponify fat. The third large class of chemicals used for this purpose includes the halogen salts. These are usually used in hypertonic solutions, but still sufficiently dilute so that there is considerable dissociation. The basic radical, as before, can saponify the fat. But the halogen radical is the more important, for that can saturate the acid, which no longer inhibits enzyme action after saturation (Jobling and Petersen). The compounds of bromine and iodine are more effective than the chlorides as parthenogenetic agents, just as they more easily saturate fatty acids. Such a solution must be hypertonic to allow the salt to penetrate a membrane tuned to normal sea-water. Finally it must not be forgotten that lipolysin is both a fat solvent and a parthenogenetic agent.

That these parthenogenetic agents act by removing an inhibitor is indicated by experiments of R. S. Lillie ('12), who found that 'resistant' *Asterias* eggs at the close of the season would give a much greater percentage of cleavages if treated with a very dilute solution of lipoid solvent or halogen compound before fertilization with sperm.

Early in June, 1915, I found few *Asterias* eggs mature, and those in which the germinal vesicle had broken down failed to attract sperm in any visible numbers. Some of these eggs were treated with solutions of lipoid solvents and other compounds (tables 14 and 15). In the treated eggs, mature ova and cleavages were subsequently found in much larger percentages than in the controls. The treatment not only enabled the sperm to fertilize more mature eggs, but enabled more eggs to form polar bodies.

Sperm to which had been added resistant *Asterias* eggs were neither more nor less active than before and did not collect around the eggs. They became very active, however, if the eggs had

been previously treated for one hour with 0.3 per cent solution of ether in sea-water and then washed thoroughly. Moreover, from four out of eleven females tested, the treated eggs, whether mature or not, became surrounded by dense halos of sperm (see

TABLE 14
The effect of lipoid solvents on resistant Asterias eggs

	A		B ¹		C		D		E	F		G		H
	Control	Inseminated	Control	Inseminated	Control	Inseminated	Control	Inseminated	Control	Control	Inseminated	Control	Inseminated	Control
Untreated controls.....	9		8		1	1	0	1	0	1	0	0	2	0
Ether, 0.3 per cent														
60 minutes.....	0	46	0	50	2	56	0	37	0	17	0	20	0	15
90 minutes.....	0	44	0	50										
Chloroform, saturated														
35 minutes.....	0	53	0	50										
48 minutes.....	0	46	0	50										
Chloroform, one-fourth saturated														
45 minutes.....	0	39	0	40										
60 minutes.....	0	26	0	40										
75 minutes.....	0	20	0	20										
Chlorethane, 0.1 per cent														
35 minutes.....	0	48	0	25										
55 minutes.....	0	36	0	25										
90 minutes.....	0	25	0	25										
Chloral hydrate, 0.1 per cent														
50 minutes.....	0	54	0	55										
2 hours.....	0	44	0	50										
3 hours.....	0	7	0	Few										
Butyric acid, 0.5 cc. $\frac{N}{10}$ + 50 cc. sea-water														
12 minutes.....	0	45	0	50										
20 minutes.....	0	48	0	50										
40 minutes.....	0	46	0	50										

¹ Estimated.

photographs). The others were unaffected. When, late in the season, Asterias eggs were again resistant, this experiment was repeated. Out of ten batches of eggs treated, every one attracted sperm decidedly better than the control.

Since the inhibitor present in the egg is a compound of an unsaturated fatty acid, iodine should saturate the acid and enable the egg to develop. The validity of this reasoning is shown by experiments in which Miss Hague and the writer ('17) used iodine as a parthenogenetic agent with *Arbacia* eggs. The curves in fig. 2 and the data in table 16 summarize the results by

TABLE 15

The effect of salt solutions on resistant Asterias eggs. The amount of reagent indicated in the first column was added to 100 cc. sea-water. The eggs were treated with these solutions for twenty minutes, washed, and inseminated

	PERCENTAGE OF CLEAVAGES					
	A		B		C	D
	Control	Inseminated	Control	Inseminated	Inseminated	Inseminated
Untreated controls.....	0	4	1	11	5	25
Calcium chloride						
4 cc. 2.5 molecular.....	20	24			22	
2 cc. 2.5 molecular.....	10	38			15	
1 cc. 2.5 molecular.....			1	7		18
5 cc. 2.5 molecular.....			1	7		9
Lithium chloride						
8 cc. normal.....	6	36			16	
4 cc. normal.....	3	53			19	
2 cc. normal.....			1	9		31
1 cc. normal.....			1	8		25
Potassium iodide						
4 cc. normal.....	3	48			26	
2 cc. normal.....	1	47			23	
1 cc. normal.....			1	10		25
0.5 cc. normal.....			1	10		15

Note.—The addition of very dilute KCNS (about 1 cc. of a 1 per cent solution to 100 cc. of sea-water) likewise produced a great increase in the number of eggs fertilized.

giving the average number of cleavages obtained by each method of treatment. The fact that the length of time during which the eggs are subjected to iodine does not affect the number of cleavages, indicates that the reagent not only enters the egg immediately, but also affects it immediately to its fullest extent. This strengthens the idea that iodine accomplishes its result by combining with the unsaturated fatty inhibitor. The membranes

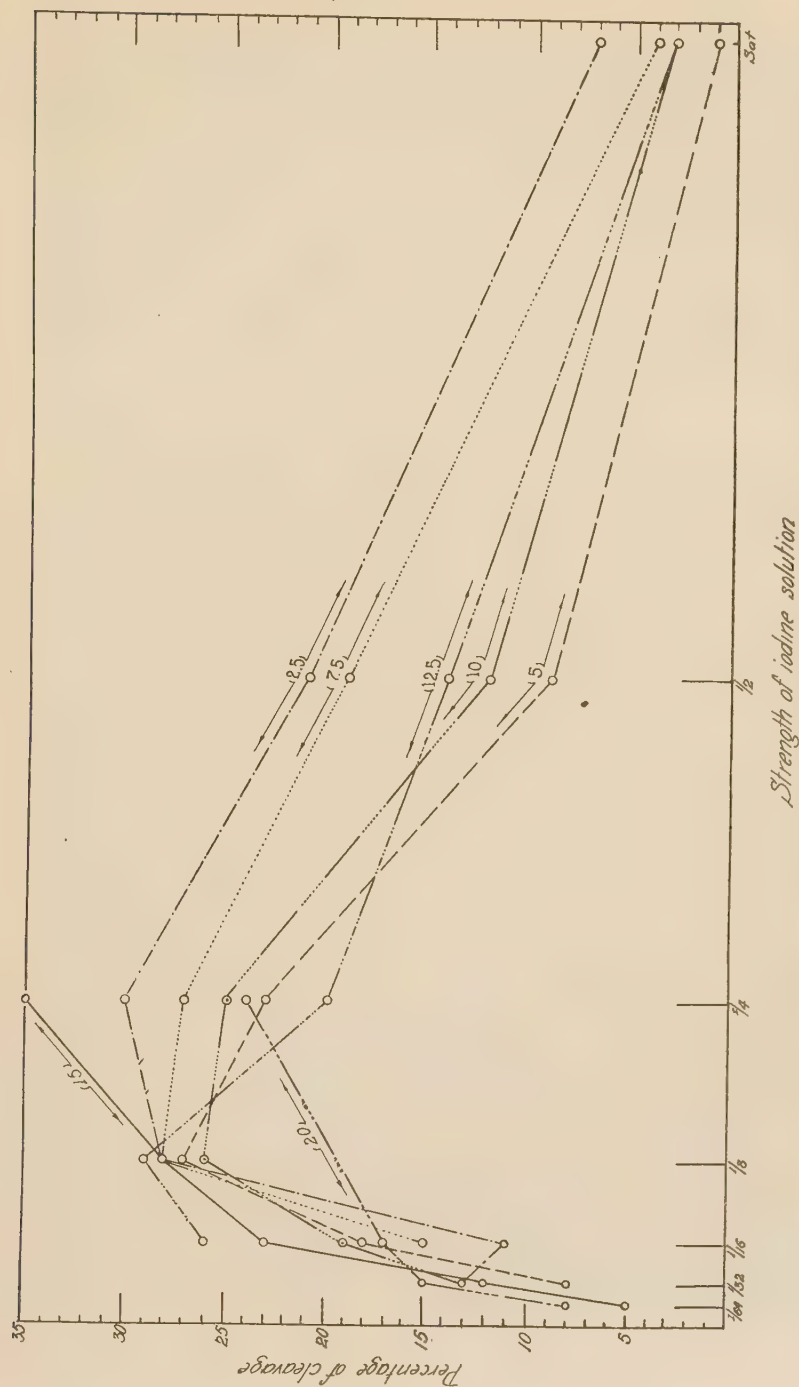


Fig. 2 Curves showing the percentage of changes obtained by using iodine as a parthenogenetic agent. The numbers on the curves indicate the number of minutes during which the eggs were exposed to iodine.

formed on these eggs were remarkable in resembling the membranes on eggs fertilized with sperm more closely than those on the usual parthenogenetic eggs—another reason for the belief that iodine reacts with the normal inhibitor and so permits the normal activator to function.

Arbacia eggs, like those of Asterias, become resistant to fertilization toward the close of the breeding season. Their fertility may likewise be increased by allowing them to stand in dilute iodine solution ten minutes before insemination. It should be noted that iodine treatment, like any other form of 'doctoring,'

TABLE 16
The parthenogenetic effect of iodine

TIME	SATURATED IODINE SOLUTION		$\frac{1}{2}$ SATU-RATED IODINE SOLUTION		$\frac{1}{4}$ SATU-RATED IODINE SOLUTION		$\frac{1}{8}$ SATU-RATED IODINE SOLUTION		$\frac{1}{16}$ SATU-RATED IODINE SOLUTION		$\frac{1}{32}$ SATU-RATED IODINE SOLUTION		$\frac{1}{64}$ SATU-RATED IODINE SOLUTION		CONTROLS	
		+ Hypertonic		+ Hypertonic		+ Hypertonic		+ Hypertonic		+ Hypertonic		+ Hypertonic		+ Hypertonic		+ Hypertonic
<i>minutes</i>																
2.5	2	7	15	21	21	30	14	28	9	11	8	13			4	6
5.0	1	1	15	9	22	23	19	27	11	18	6	8			3	5
7.5	7	4	18	19	29	27	26	28	6	15					2	5
10.0	3	3	6	12	24	25	20	26	14	19	4	13			3	6
12.5	11	3	9	14	18	20	16	29	16	26					3	4
15.0					23	35	17	28	15	23	10	12	2	5	3	5
20.0					21	24	9	5	15	17	14	15	2	8	4	5
25.0									15	20	8	16			7	9

does not improve eggs that are already fairly normal, like those in the last column of table 17.

In this connection it is interesting to recall the experiment of E. P. Lyon and L. F. Shackell ('10). They found that iodine, as indicated by the starch reaction, disappeared more rapidly from sea-water in the presence of unfertilized eggs than of eggs which had been fertilized. From this they concluded that the unfertilized eggs are more permeable to iodine than the fertilized. In the light of my own experiments, it seems more probable that the unfertilized eggs contained more unsaturated fatty acid with which the iodine might combine.

On the other hand, if the eggs be allowed to settle in test-tubes and only the supernatant fluid be tested, it will be found that the liquid above the unfertilized eggs combines less readily with iodine than does that above the fertilized eggs. The first inference from this was that the fertilized eggs had excreted unsaturated fatty acid into the sea-water. If, however, we add to the water poured off from unfertilized eggs an amount of sperm suspension equal to that present in the other tube, we find that iodine is absorbed in equal amounts from each. Therefore, the results were due to the presence of sperm in the water above fertilized eggs, rather than to unsaturated acid excreted at the time of fertilization.

TABLE 17

The effect of iodine on resistant or overripe Arbacia eggs

	PER CENT		
1. 10 cc. eggs + sperm, control.....	24	29	81
2. 10 cc. eggs + 1 drop saturated iodine 10 minutes + sperm.....	28	39	40
3. 10 cc. eggs + 2 drops $\frac{1}{4}$ saturated iodine 10 minutes + sperm.....	36	44	62
4. 10 cc. eggs + 1 drop $\frac{1}{4}$ saturated iodine 10 minutes + sperm.....	50	48	61
5. 10 cc. eggs + 1 drop $\frac{1}{8}$ saturated iodine 10 minutes + sperm.....		31	

Neither *Asterias* nor *Arbacia* lipolysin seems to combine with iodine. The chemical parthenogenetic agents, however, react with the fatty inhibitor, and, as we saw, the egg secretion, one of the three physiological activators, also combines with it to produce a compound soluble in water.

According to this view, then, the resting egg contains enzymes which control metabolism, unsaturated fatty acid which inhibits enzyme action, and lipolysin, which reacts with the unsaturated fatty acid to make it innocuous. We can conceive that the relative amounts of these substances may change from time to time in the same egg and may differ in different eggs. If there is more than sufficient fatty acid present to combine with all the lipolysin, the surplus will inhibit the action of the enzymes and the egg

will lie dormant. As the egg 'ripens,' however, it secretes more and more lipolysin. If this accumulates within the egg to such an extent that the inhibitor is relatively greatly reduced, the metabolic enzymes become extremely active and development or cytolysis results. Perhaps natural parthenogenesis, such as occurs in *Daphnia* and aphids is the result of such conditions. The membrane of starfish and sea-urchin eggs is easily permeable to lipolysin, so that it does not normally accumulate within the egg. If, however, the eggs are crowded in a small amount of sea-water or, which amounts to the same thing, are placed in a bath of lipolysin, the loss of this substance from the egg is prevented. It therefore reacts with the inhibitor, and the egg develops—a case of autoperthenogenesis.

Any other method which disturbs the ratio of activating enzymes to fatty acid so that the proportion or effectiveness of the enzymes is increased must lead to development or cytolysis. As already stated, many chemicals have this effect, and produce parthenogenesis.

B. Activation by sperm

Can the action of the spermatozoön be explained along similar lines? Its effect probably varies with the group of animals. Ostwald ('07) measured the amount of peroxidase and catalase in 1) extracts of Amphibian eggs; 2) extracts of sperm; 3) a mixture of the two extracts. He found that the mixture contained considerably more enzyme than the sum of (1) and (2). Hence we may conclude that in Amphibia the sperm carries into the egg something which changes pro-enzymes into enzymes. It increases the activator. On the other hand, Amberg and Winternitz ('11) were unable to find any more oxidase or catalase in echinoderm eggs laked after fertilization than in those laked before. Moreover, Warburg ('08) and Loeb and Wasteneys ('13) found the same rate of oxidation in parthenogenetic as in fertilized sea-urchin eggs. Since, in this group, fertilization does not increase the amount of oxidase in the egg, the spermatozoön may act by reducing the amount or effect of the inhibitor.

In conclusion, I wish to express my gratitude to Dr. O. C. Glaser, who suggested this problem and directed the work; to Dr. F. R. Lillie for the privileges of the Marine Biological Laboratory, Woods Hole, and to the several colleagues who kindly allowed me to demonstrate to them various reactions.

IV. SUMMARY

In confirmation of the work of Lillie and Glaser, it was found that *Asterias* and *Arbacia* eggs secrete into the supernatant seawater a substance which causes the sperm of the same species to be activated, aggregated, reversibly agglutinated, and paralyzed. The secretion is also a parthenogenetic agent.

Further study of its physiological properties brought out:

1. That its presence is necessary for the fertilization of the egg, since,

a. Immature eggs of *Asterias*, which cannot be fertilized, produce a secretion with less than one-sixtieth the agglutinating power of that produced by the same eggs when mature.

b. Eggs from which the secretion has been washed do not develop when inseminated. If, however, secretion be added before insemination, they develop.

c. *Arbacia* eggs which are 'resistant' to fertilization late in the season, also produce little secretion. They fertilize normally if secretion is added.

2. That the secretion has a dual nature, as shown by the following facts:

a. It reacts with both the sperm and the egg.

b. Boiling destroys its value as a parthenogenetic agent, but not as an agglutinin.

c. Perivisceral fluid of the same species inhibits autoparthenogenesis, but not agglutination.

A qualitative study of the chemical properties of the secretion confirmed Glaser's observations, and indicated:

1. That it does not dialyse through a collodion sac, and so is probably colloidal.

2. That it contains carbon and nitrogen, but gives no clear response to protein tests. It gives a faint yellow color, in the

xanthoproteic test, however, which indicates the presence of tyrosine, phenylalanine, or tryptophane.

3. That two substances can be precipitated from the same secretion:

a. By saturation with $(\text{NH}_4)_2\text{SO}_4$, a sperm agglutinin is thrown down.

b. By a method of Robertson and others, using BaCl_2 and acetone, a parthenogenetic agent is obtained.

In an attempt to learn whether or not enzymes are present, it was found that the agglutinin resembles an enzyme in the effect upon it of x-radiation. It also follows the law of Schütz and Borissow, i.e., rate of action = $K\sqrt{C}$ ferment. The secretion does not give a positive reaction to tests for oxidase, catalase, or a proteolytic enzyme. Since the parthenogenetic agent dissolves a fat obtained from the eggs, it may contain a lipase. Hence it was called, provisionally, a lipolysin.

A study of methods of inhibiting the initiation of cleavage brought out that both autoparthenogenesis and sperm fertilization are inhibited by:

1. Asterias and Arbacia serum, which may, like mammalian sera contain antiferments specifically protecting the cells of the organism itself.

2. 'Purple x' and 'Salmon x,' substances obtained by boiling the testicular or ovarian tissues of Arbacia and Asterias.

3. Antifertilizin, obtained from eggs which have been freed from fertilizin. This inhibitor may be identical with

4. An unsaturated fatty acid obtained by extracting the eggs with ether.

Parthenogenetic agents fall into three classes: 1) The fat solvents, including lipolysin, ether, chloroform, butyric acid, etc. 2) The halogen compounds—iodine and various salts. 3) Physical methods, which may change the physical or quantitative relations of substances within the egg. Not only do these agents produce development in normal, uninseminated eggs, but they change resistant Asterias and Arbacia eggs so that they may be fertilized.

A consideration of the above facts makes it seem probable that the factors tending to produce development in the resting egg are of the nature of enzymes. The action of these, Jobling found, may be inhibited by unsaturated fatty acids. The egg remains in the resting stage so long as the action of these enzymes is inhibited by the unsaturated fatty acid. The egg itself, when mature and in a suitable medium, produces a lipolysin which binds this inhibitor. The efficiency of the inhibitor may also be lowered by physical and chemical means. In some groups, the spermatozoon appears to bind the inhibitor, in others, to increase the activity of the enzymes.

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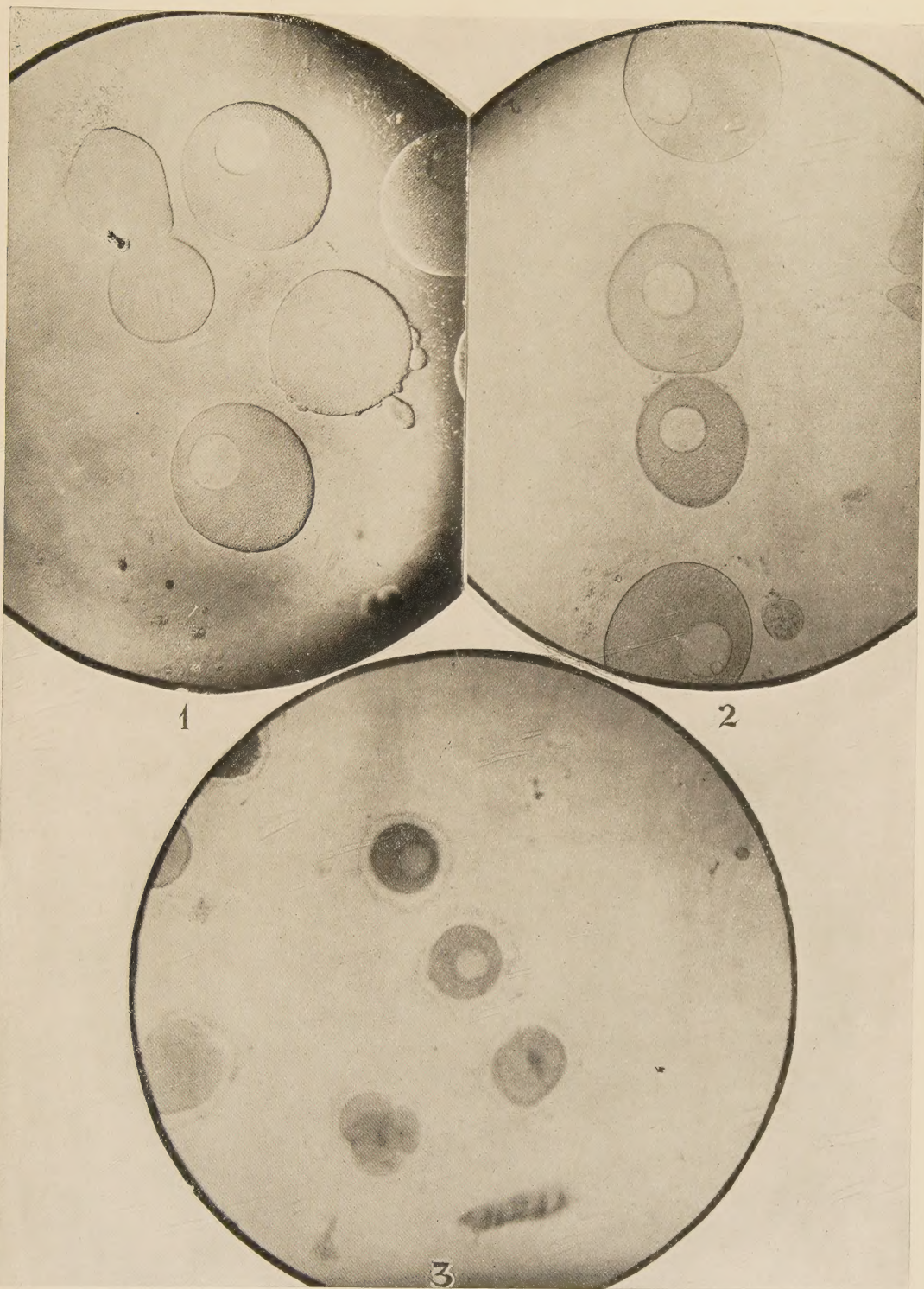
PLATE 1

EXPLANATION OF FIGURES

Photomicrographs of inseminated *Asterias* eggs:

1 and 2 Controls. Note that the spermatozoa are scattered. This shows especially well in the right hand part of 1.

3 Eggs from the same female treated for one hour with 0.3 per cent ether in sea-water, washed, and then inseminated, with the same sperm suspension as above. Note the spermatozoa in halos around both mature and immature eggs.



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